Practitioner's Docket No. U 012799-1

PATENT

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Anand C. BURMAN, et al.

Group No.: 1646

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For: BOMBESIN ANALOGS FOR TREATMENT OF CANCER

Assistant Commissioner for Patents
Washington, D.C. 20231

TECH CENTER 1500/2500

TRANSMITTAL OF CERTIFIED COPY

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: INDIA

Application Number: 147/DEL/2000

Filing Date: February 24, 2000

WARNING: *"When a document that is required by <u>statute</u> to be certified must be filed, a copy, including a photocopy or facsimile transmission of the certification is not acceptable." 37 C.F.R. 1.4(f) (emphasis added).*

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(Transmittal of Certified Copy—page 1 of 2) 5-4

SIGNATURE OF PRACTITIONER

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I the undersigned being an officer duly authorised
in accordance with the provision of the Patent Act, 1970
hereby certify that annexed hereto is a true copy of the
application form and the Complete specification filed in
connection with Patent Application No. 147/Del/2000
dated 24.02.2000.

Witness my hand this 18th day of October, 2000

(H.C. BAKSHI)
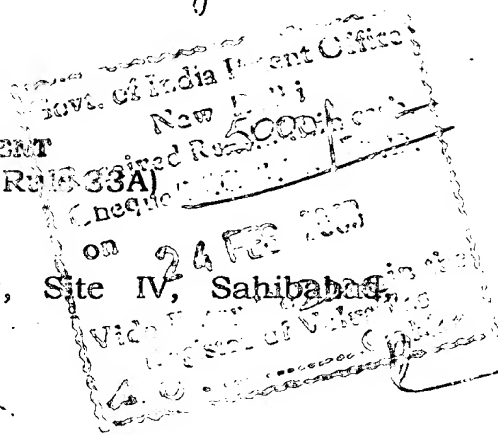DEPUTY CONTROLLER OF PATENTS & DESIGNS.

THE PATENTS ACT, 1970

APPLICATION FOR GRANT OF PATENT
(See Sections 5(2), 7, 54 and 135 and Rule 33A)

(1) We, **Dabur Research Foundation**, of 22, Site IV, Sahibabad, Ghaziabad 201 010, Uttar Pradesh, India,

(2) hereby declare –

(a) That we are in possession of an invention titled

"BOMBESIN ANALOGS FOR TREATMENT OF CANCER"

(a) that the Complete Specification relating to this invention is filed with this application;

(b) that there is no lawful ground of objection to the grant of a patent to us.

(3) Further declare that the inventors for the said invention are:

(1) BURMAN, Anand C.; (2) PRASAD, Sudhanand; (3) MUKHERJEE, Rama; (4) JAGGI, Manu; (5) SINGH, Anu T., (6) MATHUR, Archna; all of Dabur Research Foundation, of 22, Site IV, Sahibabad, Ghaziabad 201 010, Uttar Pradesh, India,

(4) We claim priority from the application filed in the following convention country, particulars of which are as follows:

NIL

(5) That we are the assignees of the true and first inventors.

(6) That our address for service in India is as follows:

SUBRAMANIAM, NATARAJ & ASSOCIATES
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(7) Following declaration was given by the inventors:

We, (1) BURMAN, Anand C.; (2) PRASAD, Sudhanand; (3) MUKHERJEE, Rama; (4) Jaggi, Manu; (5) SINGH, Anu T., (6) MATHUR, Archna; all of Dabur Research Foundation, of 22, Site IV, Sahibabad, Ghaziabad 201 010, Uttar Pradesh, India, the true and first inventor for this application declare that the applicants herein are our assignees.

_____
Anand C. BURMAN

_____
Sudhanand PRASAD

_____
Rama MUKHERJEE

_____
Anu T. SINGH

_____
Manu JAGGI

_____
Archna MATHUR

(8) That to the best of our knowledge, information and belief the facts and matters stated herein are correct and there is no lawful ground of objection to the grant of patent to me/us on this application.

(9) Following are the attachments with this application:
  (a)   Complete specification in triplicate
  (b)   Application forms 1 in triplicate
  (c)   Statement and Undertaking on FORM 3 in duplicate
  (d)   Drawings in triplicate
  (e)   Abstract

Fee Rs. .... in Cash/Cheque/Bank Draft Bearing No................ dated...............on ....Bank.

We request that a patent be granted to us for the said invention.

Dated this 24th day of February 2000

(CH. SUBRAMANIUM)
Attorney for
DABUR RESEARCH FOUNDATION
Signature with designation

The Controller of Patents
The Patent Office,
At New Delhi

Form 2

THE PATENTS ACT, 1970

COMPLETE SPECIFICATION
(Section 10)

"BOMBESIN ANALOGS FOR TREATMENT OF CANCER"

We, **Dabur Research Foundation,** of 22, Site IV, Sahibabad, Ghaziabad 201 010, Uttar Pradesh, India,

The following specification particularly describes and ascertains the nature of the invention and the manner in which it is to be performed:

BOMBESIN ANALOGS FOR TREATMENT OF CANCER

FIELD OF INVENTION

The present invention encompasses the novel peptides that are antagonists to bombesin and bombesin like peptides and useful in the treatment of cancer. The invention particularly relates to the design and synthesis of the novel peptides incorporating α,α - amino acids in a site specific manner.

BACKGROUND OF THE INVENTION

Bombesin is a 14 amino acid peptide which was first isolated from the skin of the frog Bombina bombina (Anastasi et al., Experientia, 1971, 27, 166) and has the sequence:

pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂

Gastrin releasing peptide(GRP) is a 27 amino acid peptide isolated from the porcine gut. The last ten amino acids at the C-terminus of gastrin releasing peptide correspond with one amino acid alteration (3) to the last ten amino acids of bombesin, viz: H-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ .It has been reported (J. H. Walsh and J. R. Reeve, Peptides 6, (3), 63-68, (1985) that bombesin and bombesin-like peptides such as gastrin releasing peptide (GRP) are secreted by human small-cell lung cancer (SCLC) cells. It has been postulated (P. J. Woll and E. Rozengurt, PNAS 85, 1859-1863, (1988)) that gastrin releasing factor antagonists would bind competitively to bombesin receptors in animals and would therefore be of use in the treatment of SCLC and/or in the control of clinical symptoms associated with this disease and due to hypersecretion of this peptide hormone. Analogues of bombesin/ GRP have been shown to inhibit the binding of gastrin releasing peptide to a SCLC cell line and to inhibit the growth of SCLC cells in-vitro and in-vivo (S. Mahmoud et al., CancerResearch, 1991,51, 1/798; Moody TW et al., Life Sci., 1995, 56,521; Moody TW et al., Peptides, 1996, 17, 1337).After Bombesin/GRP cell receptors were established on SCLC cells, receptors were also found to be present on human prostate cells. Reile H et al., (Prostate, 1994, 25: 29-38) showed tht the PC-3 and DU-145 human prostate cancer cell lines possess specific high- affinity receptors for bombesin/ GRP and are suitable models for the evaluation of anti-neoplastic activity of new bombesin/ GRP antagonists in the treatment of androgen- dependent prostate cancer. Bombesin also increased the penetration of the two human prostatic carcinoma cell

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lines, the relatively indolent LNCaP cells and the aggressively growing and invasive PC-3 cells, in an *in vitro* invasion of reconstituted basement membrane (Matrigel)(Hoosein NM et al., J Urol, 149(5): 1209-1213).High- affinity binding sites for GRP were found on human colorectal cancer tissue(Preston, SR. et al, Br. J. Can.,1995, 71, 1087), suggesting that bombesin- like peptides may have a role in the pathogenesis of colorectal cancer, and bombesin receptor antagonists may be of value in the treatment of receptor- positive tumours. Inhibitory effects of bombesin/ GRP antagonist RC-3095 and somatostatin analogue RC-160 were also seen on growth of HT-29 human colon cancer xenografts in nude mice (Radulovic S et al., Acta Oncol, 1994, 33(6): 693-701).

Studies with the anti-bombesin/GRP antibodies lead to the hypothesis that it may be possible to disrupt the autocrine growth cycle of bombesin/GRP using designed peptidereceptor antagonists. Since then several types of Bombesin antagonist have been reported. These antagonist have been defined by type and position of the substitutions of the natural sequence. Early receptor antagonist suffered from low potency, lack of specificity, and toxicity, which presented serious problems with their scientific and therapeutic use.

More recent work has concentrated on modification of the carboxy terminal (C-terminal) region of these peptides to interrupt the receptor interaction utilizing a variety of different types of C-terminal modified analogs. These have included incorporation of D-amino acids, non-peptide bonds for example (.psi.>CH.sub.2 NH!), amide, and ester modifications. These alterations gave rise to certain peptides having improved characteristics (Staley J et al., Peptides, 1991, 12(1): 145-9; Coy DH et al., J Natl Cancer Inst Monogr, 1992,13: 133-9). Other patents that describes bombesin and related analogs are:

USP   5,834,433 (1998)

USP   5,723,578 (1998)

USP   5,620,959 (1997)

USP   5,620,955 (1997)

USP   5,428,019 (1995)

USP   5,369,094 (1994)

USP   5,084,555 (1992)

A Bombesin/ GRP antagonist (RC-3940-II) was found to inhibit the proliferation of SW-1990 human pancreatic adenocarinoma cells in vivo and in vitro (Qin, Y. et al.,1995, Int. J. Cancer, 63, 257). Similar effect was seen with bombesin/ GRP antagonist RC-3095 on the growth of CFPAC-1 human pancreatic cancer cells transplanted to nude mice or cultured in vitro (Qin Y et al., Can Res, 1994, 54(4): 1035-41).

As reported earlier, the autocrine growth cycle of Bombesin/ GRP in SCLC can be disrupted by BBN/GRP antagonists such as [Psi 13,14]BBN. Several BBNanalogues were solid phase synthesized and incubated with intact SCLC cells at 37°C in RPMI medium in a time course fashion (0-1080 minutes) to determine enzymatic stability.The proteolytic stability of the compounds was determined by subsequent HPLC analysis.[Psi 13,14]BBN was found to be very stable to metabolic enzymes (T1/2= 646 min.) and inhibited SCLC xenograft formation in vivo in a dose- dependent manner (Davis TP et al., Peptides, 1992, 13(2): 401-7).

Female athymic nude mice bearing xenografts of the MCF-7 MIII human breast cancer cell line were treated for 7 weeks with bombesin/ GRP antagonist (D-Tpi6, Leu13 psi[CH2NH]-Leu14) bombesin (6-14)(RC-3095) injected subcutaneously daily at a dose of 20 μg and LHRH antagonist SB-75 (Cetrorelix) administered biweekly in the form of microgranules releasing 45μg/ day. After 2 weeks of treatment, a significant inhibition of tumour volume was observed in the groups treated with RC-3095 alone or in combination with SB-75 (Yano T et al., Cancer, 1994, 73(4): 1229-38).

Pinski J et al., (Int. J. Cancer, 1994, 57(4): 574-580), demonstrated for the first time that the growth of gastrin- responsive human gastric carcinoma MKN45 cell line xenografts in nude mice could be inhibited not only by somatostatin analogues, but also by administration of modern bombesin/ GRP antagonists, such as RC- 3095, or a combination of these. RC-3095 also effectively inhibited tumour growth in nude mice bearing xenografts of the human gastric cancer cell line Hs746T (Qin Y et al., J Cancer Res Clin Oncol, 1994,120(9):519-528).

This invention describes the preparation and use of peptide analogs of bombesin/ GRP using constrained amino acids and their use thereof for cancer therapy, alone, or in combination or as an adjunct to cancer chemotherapy.

The design of conformationally constrained bioactive peptide derivatives has been one of the widely used approaches for the development of peptide-based therapeutic agents. Non-standard amino acids with strong conformational preferences may be used to direct the course of polypeptide chain folding, by imposing local stereochemical constraints, in de novo approaches to peptide design. The conformational characteristics of α, α-dialkylated amino acids have been well studied. The incorporation of these amino acids restricts the rotation of ϕ, ψ angles, within the molecule, thereby stabilizing a desired peptide conformation. The prototypic member of α,α-dialkylated aminoacids, α-aminoisobutyric acid (Aib) or α,α-dimethylglycine has been shown to induce β-turn or helical conformation when incorporated in a peptide sequence (Prasad and Balaram, 1984, Karle and Balaram, 1990). The conformational properties of the higher homologs of α,α-dialkylated amino acids such as di-ethylglycine (Deg), di-n-propylglycine (Dpg) and di-n-butylglycine (Dbg) as well as the cyclic side chain analogs of α,α-dialkylated amino acids such as 1-aminocyclopentane carboxylic acid (Ac5c), 1-aminocyclohexane carboxylic acid (Ac6c), as 1-aminocycloheptane carboxylic acid (Ac7c) and as 1-aminocyclooctane carboxylic acid (Ac8c) have also been shown to induce folded conformation (Prasad et al., 1995 ; Karle et al., 1995). α,α-dialkylated amino acids have been used in the design of highly potent chemotactic peptide analogs (Prasad et al., 1996)

SUMMARY OF INVENTION

The present invention comprises of polypeptides of the following general formula.

D-Phe-Gln-R1–R2 -Val-R3 -His-R4 -NH_2

wherein   R1=Trp or D-Trp

            R2= Ala or Aib or Deg

            R3 = Gly or Aib or Deg or Dpg or Ac5c

            R4= Leu or Ile

or a hydrolyzable carboxy protecting group; or pharmaceutically acceptable salt thereof.

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The present invention also envisages methods of treatment using the polypeptides of the present invention, pharmaceutical compositions comprising of such polypeptides and processes for their preparation. These peptides possess antagonist properties against bombesin and bombesin- like peptides and are useful in the treatment of malignant diseases.

DETAILED DESCRIPTION OF THE INVENTION

The novel peptide analogs embodied in the present invention contain amino acids, namely α, α-dialkylated amino acids, which have been known to induce highly specific constraints in the peptide backbone. The α, α-dialkylated amino acids, used in the present invention are synthesized from the corresponding ketones. In a preferred embodiment of the invention, the ketones are first converted into the corresponding hydantoins which are hydrolyzed to yield the aforesaid amino acids. In a preferred embodiment of the present invention, 60% sulphuric acid has been employed as the hydrolyzing agent.

The novel peptides in the present invention have been generated by a using solid phase techniques or by a combination of solution phase procedures and solid phase techniques or by fragment condensation. Although these methods for the chemical synthesis of polypeptides are well known in the art (Stewart and Young, 1969), the use of solid phase methodology for the synthesis of peptides incorporating α, α-dialkylated amino acids, is not known in the prior art.

In a preferred embodiment of the present invention the peptides were synthesized using the Fmoc strategy, on a semi automatic peptide synthesizer (CS Bio, Model 536), using optimum side chain protection. The peptides were assembled from C-terminus to N-terminus. Peptides amidated at the carboxy-terminus were synthesized using the Rink Amide resin. The loading of the first Fmoc protected amino acid was achieved via an amide bond formation with the solid support, mediated by Diisopropylcarbodiimide (DIPCDI) and HOBt. Substitution levels for automated synthesis were preferably between 0.2 and 0.6 mmole amino acid per gram resin.

The resin employed for the synthesis of carboxy-terminal amidated peptide analogs was 4-(2', 4'-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-derivatized polystyrene 1% divinylbenzene

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(Rink Amide) resin (100-200 mesh), procured from Calbioichem-Novabiochem Corp., La Jolla, U.S.A., (0.47 milliequivalent NH. sub. 2 /g resin).

The N-terminal amino group was protected by 9-flourenylmethoxycarbonyl (Fmoc) group. Trityl (trt) or t-butyloxycarbonyl (Boc) were the preferred protecting groups for imadazole group of Histidine residue. The hydroxyl groups of Serine, Threonine and Tyrosine were preferably protected by t-butyl group (tBu) 2,2,5,7,8-pentamethyl-chroman-6-sulfonyl (Pmc) or 2,2,4,7,-pentamethyl-dihydrobenzenofuran-5-sufonyl (Pbf) were the preferred protecting groups for the guandino group of Arginine. Trityl was the preferred protecting group for Asparagine and Glutamine and tertiary butyl group (tBu) was the preferred protecting group for Aspartic acid and Glutamic acid. The tryptophan residue was either left unprotected or used with Boc protection. The side chain amino group of Lysine was protected using Boc group preferrably.

In a preferred embodiment of the invention, 2-8 equivalents of Fmoc protected amino acid per resin nitrogen equivalent were used. The activating reagents used for coupling amino acids to the resin, in solid phase peptide synthesis, are well known in the art. These include BOP, PyBOP, HBTU, TBTU, PyBOP, HOBt. Preferably, DCC or DIPCDI / HOBt or HBTU/HOBT and DIEA were used as activating reagents in the coupling reactions.

The protected amino acids were either activated *in situ* or added in the form of preactivated esters known in the art such as NHS esters, Opfp esters etc.

The coupling reaction was carried out in DMF, DCM or NMP or a mixture of these solvents and was monitored by Kaiser test [Kaiser *et al.*, Anal. Biochem., 34, 595-598 (1970)]. In case of a positive Kaiser test, the appropriate amino acid was re-coupled using freshly prepared activated reagents.

After the assembly of the peptide was completed, the amino-terminal Fmoc group was removed and then the peptide-resin was washed with methanol and dried. The peptides were then deprotected and cleaved from the resin support by treatment with trifluoroacetic acid, crystalline phenol,

ethanedithiol, thioanisole and de-ionized water for 1.5 to 5 hours at room temperature. The crude peptide was obtained by precipitation with cold dry ether, filtered , dissolved, and lyophilized.

The resulting crude peptide was purified by preperative high performance liquid chromatography (HPLC) using a LiChroCART® C_{18} (250. Times. 10) reverse phase column (Merck, Darmstadt, Germany) on a Preparative HPLC system (Shimadzu Corporation, Japan) using a gradient of 0.1% TFA in acetonitrile and water . The eluted fractions were reanalyzed on Analytical HPLC system (Shimadzu Corporation, Japan) using a C18 LiChrospher®, WP-300 (300 X 4) reverse- phase column. Acetonitrile was evaporated and the fractions were lyophilized to obtain the pure peptide. The identity of each peptide was confirmed by electron-spray mass spectroscopy.

Synthesis of peptides

A peptide of the present invention can be made by exclusively solid phase techniques, by partial solid phase / solution phase techniques and fragment condensation. Preferred, semi-automated, stepwise solid phase methods for synthesis of peptides of the invention are provided in the examples discussed in the subsequent section of this document.

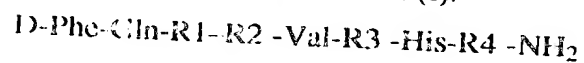Accordingly, the present invention provides a novel peptide of the following general formula
D-Phe-Gln-R1–R2 -Val-R3 -His-R4 -NH₂
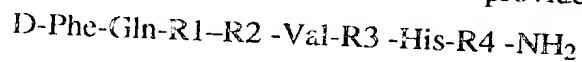
wherein   R1=Trp or D-Trp

         R2= Ala or Aib or Deg

         R3 = Gly or Aib or Deg or Dpg or Ac5c

         R4= Leu or Ile

or a hydrolyzable carboxy protecting group; or pharmaceutically acceptable salt thereof.

The present invention also provides a solid phase synthesis process for the preparation of a peptide analog of the general formula (I):
D-Phe-Gln-R1–R2 -Val-R3 -His-R4 -NH₂

wherein   R1=Trp or D-Trp

         R2= Ala or Aib or Deg

         R3 = Gly or Aib or Deg or Dpg or Ac5c

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R4= Leu or Ile

which comprises sequentially loading the corresponding protected α- α-dialkylated amino acids in sequential cycles to the amino terminus of a solid phase resin, coupling the amino acids in the presence of conventional solvents and reagents to assemble a peptide-resin assembly, removing the protecting groups and cleaving the peptide from the resin to obtain a crude peptide analog.

The present invention further provides a pharmaceutical composition containing the novel peptides of the present invention as well as a method for treatment of cancer in mammals using such pharmaceutical compositions.

The present invention will be further described in detail with reference to the following examples, as will be appreciated by a person skilled in the art is merely illustrative and should not be construed as limiting. Various other modifications of the invention will be possible without departing from the spirit and scope of the present invention.

Example: 1

First loading on Rink Amide Resin

A typical preparation of the Fmoc-Leu-Rink Amide Resin was carried out using 0.5g of 4-(2',4'-Dimethoxyphenyl- Fmoc- aminomethyl) phenoxymethyl- derivatized polystyrene 1% divinylbenzene (Rink Amide) resin (0.7 mM / g) (100-200 mesh), procured from Advanced Chemtech, Louisville, KY, U.S.A., (0.7 milliequivalent NH. sub. 2 /g resin). Swelling of the resin was typically carried out in dichloromethane measuring to volumes 10-40ml /g resin. The resin was allowed to swell in methylene chloride (2 X 25 ml, for 10 min.). It was washed once in dimethylformamide (DMF) for 1 min. All solvents in the protocol were added in 20 ml portions per cycle. The Fmoc- protecting group on the resin was removed by following steps 3-7 in the protocol. The deprotection of the Fmoc group was checked by the presence of blue beads in Kaiser test. For loading of the first amino acid on the free amino (NH$_2$) group of the resin, the first amino acid, Fmoc-Leu-OH, was weighed in three to six fold excess, along with a similar fold excess of HOBt in the amino acid vessel of the peptide synthesizer. These were dissolved in dimethylformamide (A.C.S. grade) (J.T.Baker, Phillipsburg, New Jersey, U.S. A.) and activated with DIC, just prior to the addition to the resin in the reaction vessel of the peptide synthesizer. HOBt was added in all coupling reactions, especially in the case of Gln and His. The coupling reaction was carried out for

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a period ranging from 1-3 hours. The loading of the amino acid on the resin was confirmed by the presence of colorless beads in the Kaiser Test. The loading efficiency was ascertained by the increase of weight of the resin after the addition of the amino acid.

Example: 2

Synthesis of D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH$_2$ (SEQ. I.D. NO: 1)

The synthesis of SEQ. I.D. NO: 1, amidated at the carboxy- terminus, was initiated by using all of the resin loaded with Fmoc-Leu-OH as prepared in example (2) above. This was subjected to stepwise deprotection and coupling steps as in steps 1-10 of the synthesis cycle. In each coupling reaction, a two to six fold excess of amino- acid, DIC and HOBt were used. Upon completion of synthesis and removal of the N-terminal Fmoc protecting group (steps 1-6 of the synthesis cycle), the peptide- resin was washed twice with methanol, dried and weighed to obtain 0.649g. This was subjected to cleavage in a cleavage mixture consisting of trifluoroacetic acid and scavengers, ethanedithol and water for a period of 1-4 hours at room temperature with continuous stirring. The peptide was precipitated using cold dry ether to obtain ~ 330 mg of crude peptide. The crude peptide was purified on a C18 preperative reverse phase HPLC column (250X10) on a gradient system comprising of acetonitrile and water in 0.1% TFA as described previously, in the art. The prominent peaks were collected and lyophilized, reanalysed on analytical HPLC and subjected to mass spectrometry. There was a good agreement between the observed molecular weight and calculated molecular weight (Calculated Mass= 983.1; Observed Mass= 984.2). The pure peptide was then used for bioassays.

Example :3

Synthesis of D-Phe-Gln-Trp-Aib-Val-Gly -His-Leu-NH$_2$ (SEQ. I.D. NO:2)

The synthesis, cleavage and lyophilization steps were carried out as in the Example 2 above using the appropriate amino acids. The calculated mass was 969.11 and the observed mass was 970.4 .

Example :4

Synthesis of D-Phe-Gln-D-Trp-Ala-Val-Aib-His-Leu-NH$_2$ (SEQ. I.D. NO:3) ·

The synthesis, cleavage and lyophilization steps were carried out as in the Example 2 above using the appropriate amino acids. The calculated mass was 983.11 and the observed mass was 984.30.

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Example :5

Synthesis of D-Phe-Gln-Trp-Aib-Val-Gly-His-Ile -NH₂ (SEQ. I.D. NO:4)

The synthesis, cleavage and lyophilization steps were carried out as in the Example 2 above using the appropriate amino acids. The calculated mass was 969.11 and the observed mass was 970.2.

Example :6

Synthesis of D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile -NH₂ (SEQ. I.D. NO:5)

The synthesis, cleavage and lyophilization steps were carried out as in the Example 2 above using the appropriate amino acids. The calculated mass was 983.11 and the observed mass was 984.2 .

Example :7

Synthesis of D-Phe-Gln-D-Trp-Ala-Val-Dpg -His-Leu-NH₂ (SEQ. I.D. NO:6)

The synthesis, cleavage and lyophilization steps were carried out as in the Example 2 above using the appropriate amino acids. The calculated mass was 1039.29 and the observed mass was 1040.4.

Biological activity of peptide

The cytotoxicity of the peptide analog listed in the table was carried out by two day MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. MTT assay is based on the principle of uptake of MTT, a tetrazolium salt, by metabolically active cells where it is metabolized by active mitochondria into a blue colored formazon product, which can be read spectrometrically (Ref. J.of immunological Methods 65: 55-63, 1983). To prepare the MTT stock solution needed, MTT was dissolved in phosphate buffered saline with a pH of 7.4 to obtain an MTT concentration of 5 mg/ml; the resulting mixture was filtered through a 0.22 micron filter to sterilize and remove a small amount of insoluble residue. This filtered mixture was the MTT stock solution.

Briefly, for each tumour type, 10,000 cells were seeded in 96-well tissue culture plate and incubated with each peptide concentration individually in a CO₂ incubator for 48 hrs. The peptide analog at different concentrations was added once every 24 hrs during the incubation period. Control cultures, which were not treated with the peptide was similarly incubated. The assay was terminated by adding 100μg (20μl) of MTT to each well, incubating for three hours, decanting supernatant and finally adding 150 μl of dimethylsulphoxide to each well to dissolve the formazon. The plates were

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incubated for 15 minutes at 37° C and read spectrophotometrically at 540 nm; and cytotoxicity percentage was calculated by following formula:

Cytotoxicity Percentage= 100x [1-X/R1], where X= (absorbance of the treated sample at 540 nm-absorbance of a blank at 540 nm) and R1= (absorbance of the untreated control at 540nm)-(absorbance of the blank at 540nm).

Thus in each of the MTT cytotoxicity assay the percentage was calculated according to the above formula and was based on the proliferation of the untreated controls, the value of which was considered as 100%.

Example :8

The biological activity of synthesized peptide SEQ I.D:1 was tested on different human tumour cell lines such as HT-29 & PTC (colon), A549 (non small lung cell), KB (oral squamous cell), MCF7 & MDA.MB.453 (Breast), HuTu80 (duodenum), PA-1 (ovary), MOLT-4 (leukemia) and MIAPaCa2 (Pancreas) at various molar concentrations. The percentage cytotoxicity induced by different concentrations of the peptide SEQ ID: 1 is summarized in the following table.

Cell Line	Percentage cytotoxicity at different concentrations					
	1μ M	100n M	10 nM	1nM	100pM	10pM
MCF 7	Nil	Nil	24.35 ± 5	30.68 ± 6	38.95± 4.5	39.33 ± 2.6
MIAPaCa2	33.3 ± 4.5	30.3 ± 4.2	33.2 ± 6.7	36.4 ± 0.5	28.2 ± 4.5	27.4 ± 4.5
HuTu80	12.2 ± 4.0	15.5 ± 4.7	14.3 ± 3.5	13.3 ± 4.0	14.7 ± 4.2	10.3 ± 3.5
KB	32.1 ± 5.0	31.6 ± 6.5	30.9 ± 5.5	30.4 ± 6.5	26.4 ± 4.5	40.9 ± 5.5
A549	30.7 ± 6.5	23.6 ± 4.5	32.2 ± 5.5	32.4 ± 4.5	25.2 ± 3.5	30.5 ± 3.5
HT29	25.4 ± 5.5	17.8 ± 4.5	11.8 ± 5.0	20.3 ± 4.5	19.9 ± 5.5	18.7 ± 4.5
PTC	17.9 ± 2.5	27.7 ± 2.8	27.7 ± 3.6	23.8 ± 2.8	26.5 ± 3.8	80.0 ± 7.1
MDA.MB.453	5.6 ± 3.5	11.2 ± 3.1	Nil	9.6 ± 1.9	25.5 ± 2.9	49.5 ± 4.2
PA-1	31.2 ± 5.1	34.2 ± 5.8	25.4 ± 4.2	36.1 ± 6.1	40.1 ± 6.2	37.7 ± 3.9
MOLT-4	9.0 ± 1.2	1.4 ± 1.0	Nil	1.0 ± 0.4	15.9 ± 3.0	49.7 ± 4.1

Example : 9

The cytotoxic activity of other synthesized bombesin analogs was tested on eight human tumor cell lines namely HT-29, SW620, PTC (all colon), PA-1 (ovary), A549 (lung), HBL100 (breast),

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MOLT-4 (leukemia) and DU145 (prostate). The tumor cells were collected at exponential growth phase and resuspended in medium (1.5 x 10^6 cells/ml in RPMI 1640 containing 10% FBS). 150μl of medium was added to the wells of a 96-well tissue culture plate (Nunc, Denmark) followed by 30μl of cell suspension. The plate was left in incubator (37^0C, 5% CO_2) overnight. 20μl of the peptide (10^{-7} to 10^{-10}M concentration) was added to marked wells of the 96-well plate. Each concentration was plated in triplicates. 20μl of medium alone was added to control wells while wells without cells served as blanks. A total volume of 200μl was ensured in each well and plate was left in incubator (37^0C, 5% CO_2). After 72 hours of incubation an MTT assay was performed and percentage cytotoxicity was calculated with respect to control cells. Following tables show the cytotoxicity achieved on various cell lines at different concentrations.

PA-1

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	2.3 ± 2.9	4.3 ± 0.2	16.2 ± 2.9	12.6 ± 2.9
SEQ:ID:3	8.8 ± 1.9	20.9 ± 5.3	16.0 ± 3.9	25.6 ± 6.3
SEQ ID:4	9.2 ± 1.0	8.7 ± 1.9	7.4 ± 1.0	11.1 ± 2.9
SEQ ID:5	9.6 ± 4.1	22.7 ± 3.4	25.6 ± 2.9	24.5 ± 4.2
SEQ ID:6	10.4 ± 3.7	20.4 ± 3.0	23.8 ± 4.2	23.3 ± 5.5

PTC

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	9.8 ± 1.7	2.1 ± 0.2	8.7 ± 1.5	14.9 ± 1.1
SEQ:ID:3	20.4 ± 4.2	15.9 ± 2.4	23.0 ± 4.2	13.9 ± 2.2
SEQ ID:4	24.7 ± 5.2	10.4 ± 0.8	9.1 ± 0.7	10.1 ± 0.6
SEQ ID:5	9.3 + 1.8	7.6 + 0.7	12.4 + 2.1	8.2 + 0.9
SEQ ID:6	8.7 ± 2.1	5.4 ± 1.7	12.5 ± 1.7	12.3 ± 1.9

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DU145

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	24.9 ± 3.2	23.4 ± 3.3	22.8 ± 4.1	23.2 ± 3.7
SEQ:ID:3	32.3 ± 3.8	22.0 ± 3.4	10.6 ± 0.9	29.3 ± 2.9
SEQ ID:4	13.7 ± 0.9	16.6 ±	23.9 ± 5.2	12.1 ± 0.8
SEQ ID:5	NIL	NIL	ND	ND
SEQ ID:6	19.1 ± 2.1	22.5 ± 2.2	21.4 ± 6.2	28.1 ± 3.5

SW620

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	34.3 ± 4.2	23.2 ± 2.0	27.8 ± 2.8	30.4 ± 3.2
SEQ:ID:3	25.6 ± 4.2	30.1 ± 4.0	29.7 ± 4.2	38.0 ± 3.8
SEQ ID:4	23.5 ± 5.1	38.1 ± 7.3	33.5 ± 5.2	24.8 ± 4.2
SEQ ID:5	25.4 ± 2.9	20.8 ± 1.9	32.0 ± 5.8	33.6 ± 5.8
SEQ ID:6	29.4 ± 2.9	33.0 ± 3.8	20.6 ± 3.9	24.8 ± 5.2

HT29

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	38.6 ± 5.3	38.9 ± 7.3	39.6 ± 4.3	43.3 ± 4.4
SEQ:ID:3	35.7 ± 2.8	44.4 ± 4.0	27.9 ± 2.9	42.0 ± 2.0
SEQ ID:4	NIL	6.8 + 0.7	26.7 ± 4.2	16.8 ± 0.5
SEQ ID:5	15.5 + 1.9	28.2 + 2.8	ND	ND
SEQ ID:6	34.8 ± 4.2	18.9 ± 4.2	34.7 ± 3.3	21.4 ± 3.1

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MOLT4

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	16.2 ± 0.6	28.7 ± 4.2	19.3 ± 1.8	28.5 ± 4.8
SEQ:ID:3	NIL	4.3 ± 0.6	6.4 ± 0.2	8.7 ± 0.6
SEQ ID:4	NIL	20.4 ± 4.3	0.8 ± 0.1	11.0 ± 0.6
SEQ ID:5	13.1 ± 0.3	NIL	NIL	ND
SEQ ID:6	2.6 ± 0.1	12.8 ± 3.3	9.3 ± 0.2	16.6 ± 3.1

HBL

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	25.0 ± 3.1	33.2 ± 5.2	30.6 ± 4.2	33.0 ± 3.6
SEQ:ID:3	19.4 ± 4.5	16.7 ± 3.6	31.6 ± 5.3	19.3 ± 2.7
SEQ ID:4	17.0 ± 0.5	6.0 ± 0.4	1.2 ± 0.3	NIL
SEQ ID:5	16.1 ± 3.9	7.0 ± 0.7	12.0 ± 0.7	4.0 ± 0.6
SEQ ID:6	11.9 ± 2.1	14.4 ± 2.1	12.2 ± 1.9	12.1 ± 1.9

A549

S.No	Percent Cytotoxicity			
	100 nM	10nM	1 nM	100 pM
SEQ ID:2	20.0 ± 2.2	20.6 ± 1.9	22.7 ± 2.9	20.7 ± 4.2
SEQ:ID:3	30.3 ± 4.3	22.2 ± 3.1	20.2 ± 4.2	25.2 ± 5.6
SEQ ID:4	1.9 ± 0.6	3.2 ± 0.1	13.0 ± 0.8	12.4 ± 0.7
SEQ ID:5	6.7 ± 2.0	17.9 ± 0.9	ND	ND
SEQ ID:6	21.7 ± 3.3	20.7 ± 2.2	19.7 ± 3.1	17.0 ± 2.7

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We claim:

1. A peptide of the following general formula

D-Phe-Gln-R1–R2 -Val-R3 -His-R4 -NH$_2$

wherein   R1=Trp or D-Trp

              R2= Ala or Aib or Deg

              R3 = Gly or Aib or Deg or Dpg or Ac5c

              R4= Leu or Ile

or a hydrolyzable carboxy protecting group; or pharmaceutically acceptable salt thereof.

2. The peptide of the claim 1 wherein R1=Trp, R2=Ala, R3=Aib and R4=Leu, and said peptide has the formula:

D-Phe-Gln-Trp-Ala-Val-Aib-His-Leu-NH$_2$(SEQ ID NO:1)

or a pharmaceutically acceptable salt thereof.

3. The peptide of the claim 1 wherein R1=Trp, R2=Aib, R3=Gly and R4=Leu, and said peptide has the formula:

D-Phe-Gln-Trp-Aib-Val-Gly -His-Leu-NH$_2$ (SEQ ID NO:2)

or a pharmaceutically acceptable salt thereof.

4. The peptide of the claim 1 wherein R1=D-Trp, R2=Ala, R3=Aib and R4=Leu, and said peptide has the formula:

D-Phe-Gln-D-Trp-Ala-Val-Aib-His-Leu-NH$_2$ (SEQ ID NO:3)

or a pharmaceutically acceptable salt thereof.

5. The peptide of the claim 1 wherein R1=Trp, R2=Aib, R3=Gly and R4=Ile, and said peptide has the formula:

D-Phe-Gln-Trp-Aib-Val-Gly -His-Ile -NH$_2$ (SEQ ID NO:4)

or a pharmaceutically acceptable salt thereof.

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6.   The peptide of the claim 1 wherein R1=Trp, R2=Ala, R3=Aib and R4=Ile, and said peptide has the formula:

D-Phe-Gln-Trp-Ala-Val-Aib-His-Ile -NH$_2$(SEQ ID NO:5)

or a pharmaceutically acceptable salt thereof.

7.   The peptide of the claim 1 wherein R1=D-Trp, R2=Ala, R3=Dpg and R4=Leu, and said peptide has the formula:

D-Phe-Gln-D-Trp-Ala-Val-Dpg-His-Leu-NH$_2$ (SEQ ID NO:6)

or a pharmaceutically acceptable salt thereof.

8.   The peptide of the claim 1 wherein R1=Trp, R2=Deg, R3=Gly and R4=Leu, and said peptide has the formula:

D-Phe-Gln-Trp-Deg-Val-Gly -His-Leu-NH$_2$ (SEQ ID NO:7)

or a pharmaceutically acceptable salt thereof.

9.   The peptide of the claim 1 wherein R1=Trp, R2=Ala, R3=Ac5c and R4=Leu, and said peptide has the formula:

D-Phe-Gln-Trp-Ala-Val-Ac5c-His-Leu-NH$_2$(SEQ ID NO:8)

or a pharmaceutically acceptable salt thereof.

10.   An anti cancer pharmaceutical composition comprising an effective amount of a polypeptide according to claim 1, and a pharmaceutically acceptable carrier.

11.   A method of treatment of cancer in mammals which comprises the administration of an effective amount of polypeptide according to claim 1, alone or in combination of other polypeptides or anticancer molecule.

12.   A solid phase synthesis process for the preparation of a peptide analog of the general formula (I):

D-Phe-Gln-R1-R2 -Val-R3 -His-R4 -NH$_2$

wherein   R1=Trp or D-Trp

             R2= Ala or Aib or Deg

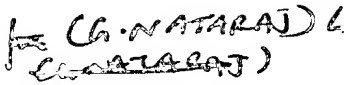   R3 = Gly or Aib or Deg or Dpg or Ac5c

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R4= Leu or Ile

which comprises sequentially loading the corresponding protected α- α-dialkylated amino acids in sequential cycles to the amino terminus of a solid phase resin, coupling the amino acids in the

presence of conventional solvents and reagents to assemble a peptide-resin assembly, removing the protecting groups and cleaving the peptide from the resin to obtain a crude peptide analog.

13.   A process as claimed in claim 12, wherein said α- α-dialkylated amino acids are protected at their α- amino groups by a 9-fluorenyl methoxy carbonyl (Fmoc) group.

14.   A process as claimed in claim 12 wherein the coupling was carried out in the presence of activated agents selected from the group consisting of BOP, PyBOP, HBTU, TBTU, TNTU, TSTU, PyBROP, HOBt.

15.   A process as claimed in claim 14 wherein the coupling was carried out in the presence of a solvent selected from the group consisting of DMF, DCM, NMP or any mixtures thereof.

16.   A process as claimed in claim 12 wherein said crude peptide is cleaved from said peptide-resin assembly by treatment with trifluoroacetic acid, crystalline phenol, ethanedithiol, thioanisole and deionised water for 1.5 to 5 hours at room temperature.

17.   A process as claimed in claim 12 wherein acids are prepared by conversion of corresponding ketones to hydantoins and hydrolysis of said hydantoins with 60 % sulfuric acid.

18.   A peptide as claimed in Claim 1 substantially as described herein and with reference to the foregoing examples.

19.   An anti cancer pharmaceutical composition 1 substantially as described herein and with reference to the foregoing examples.

20.   A method for the manufacture of a novel peptide as claimed in claim 11 substantially as described herein and with reference to the foregoing examples.

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21.   A method for treating cancer in mammals 1 substantially as described herein and with reference to the foregoing examples.

Dated this   the 24th   day   of   February   2000

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ABSTRACT

The present invention discloses a novel peptide of the following general formula

D-Phe-Gln-R1–R2 -Val-R3 -His-R4 -NH$_2$

wherein

R1=Trp or D-Trp

R2= Ala or Aib or Deg

R3 = Gly or Aib or Deg or Dpg or Ac5c

R4= Leu or Ile

or a hydrolyzable carboxy protecting group; or pharmaceutically acceptable salt thereof. These novel peptides are are antagonists to bombesin and bombesin like peptides and useful in the treatment of cancer. The present invention also encompasses a method for preparing such peptides, a pharmaceutical composition containing the novel peptides of the present invention as well as a method for treatment of cancer in mammals using such pharmaceutical compositions.

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